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HUMAN GENE THERAPY 12:629–642 (April 10, 2001)
Mary Ann Liebert, Inc.

In Vivo and *in Vitro* Correction of the *mdx* Dystrophin Gene Nonsense Mutation by Short-Fragment Homologous Replacement

ROBERT KAPSA,[1] ANITA QUIGLEY,[1] GORDON S. LYNCH,[2] KELLY STEEPER,[1] ANDREW J. KORNBERG,[1] PAUL GREGOREVIC,[2] LAWRIE AUSTIN,[1,†] and EDWARD BYRNE[1]

ABSTRACT

Targeted genetic correction of mutations in cells is a potential strategy for treating human conditions that involve nonsense, missense, and transcriptional splice junction mutations. One method of targeted gene repair, single-stranded short-fragment homologous replacement (ssSFHR), has been successful in repairing the common ΔF508 3-bp microdeletion at the cystic fibrosis transmembrane conductance regulator (CFTR) locus in 1% of airway epithelial cells in culture. This study investigates *in vitro* and *in vivo* application of a double-stranded method variant of SFHR gene repair to the *mdx* mouse model of Duchenne muscular dystrophy (DMD). A 603-bp wild-type PCR product was used to repair the exon 23 C-to-T *mdx* nonsense transition at the Xp21.1 *dys* locus in cultured myoblasts and in tibialis anterior (TA) from male *mdx* mice. Multiple transfection and variation of lipofection reagent both improved *in vitro* SFHR efficiency, with successful conversion of *mdx* to wild-type nucleotide at the *dys* locus achieved in 15 to 20% of cultured loci and in 0.0005 to 0.1% of TA. The genetic correction of *mdx* myoblasts was shown to persist for up to 28 days in culture and for at least 3 weeks in TA. While a high frequency of *in vitro* gene repair was observed, the lipofection used here appeared to have adverse effects on subsequent cell viability and corrected cells did not express dystrophin transcript. With further improvements to *in vitro* and *in vivo* gene repair efficiencies, SFHR may find some application in DMD and other genetic neuromuscular disorders in humans.

OVERVIEW SUMMARY

Short-fragment homologous replacement (SFHR) was used in this study to repair the common nonsense mutation in the dystrophin gene (*dys*) of the *mdx* mouse. SFHR was applied to cells cultured from male *mdx* mice and the possibility of gene repair efficiency improvement was investigated. Multiple application of SFHR to cells *in vitro*, combined with choice of lipofection reagent, were the primary agents by which gene conversion was achieved in 15 to 20% of *mdx* loci. Cells treated in this way were shown not to express dystrophin transcript 7 days after initiation of myotube differentiation. While the exact nature of this lack of dystrophin expression remains unclear, it is either directly or indirectly related to a significant drop in cell numbers after transfection 7 days post-differentiation. After bupivacaine-mediated muscle ablation, *in vivo* application of SFHR in four male *mdx* mice resulted in gene repair to 5×10^{-4} to 0.1% of loci in all muscles injected. Contralateral saline-injected limbs did not contain repaired *mdx* loci. If efficiency and subsequent cell viability of *in vitro* and *in vivo* gene repair may be improved, SFHR may provide a basis for treatment of many neuromuscular diseases.

INTRODUCTION

THE CAPACITY TO GENETICALLY MODIFY CELLS in a specific and controllable manner has immediate relevance to the rectification of heritable disorders in humans. Targeted genetic

[1]Melbourne Neuromuscular Research Institute and Department of Clinical Neurosciences, St. Vincent's Hospital, Fitzroy, Victoria 3065, Australia.
[2]Department of Physiology, University of Melbourne, Victoria 3010, Australia.
[†]Deceased.

modification of chromosomal loci has been used for construction of genetically modified animal models and cell lines since the 1980s (Folger *et al.*, 1982; Capecchi, 1989a; Hunger-Bertling *et al.*, 1990). Targeted gene modification by homologous recombination generally occurs at absolute targeting frequencies ranging between 5 in 10^7 (10^{-5}%) to 1 in 10^3 cells (0.1%), using microinjection, and 1 in 10^5 cells (0.001%) by electroporation (Thomas and Capecchi, 1987; Capecchi, 1989b). Such homologous recombination involves large gene-targeting vectors bearing large nonhomologous sequences for selection of recombinant cells. Gene-targeting frequency is not generally affected by the size of the nonhomologous sequence included (Mansour *et al.*, 1990). There is, however, evidence that gene-targeting efficiency is directly related to the extent of homology between the introduced and target sequences (Thomas and Capecchi, 1987). Further, one study reported a targeting efficiency of 1 in 150 cells, proposed to arise by limiting the amount of sequence disruption in the targeted gene to 20 nucleotides (Zimmer and Gruss, 1989).

Two methods of targeted gene conversion have been described, both displaying specific *in vitro* repair frequencies two or more orders of magnitude greater than traditional targeted homologous recombination. The first of these, chimeraplasty, involves introduction of chimeric RNA/DNA oligonucleotides (chimeraplasts) containing a nonmutant nucleotide locus to the mutant nuclear compartment, with consequent mismatch repair at the mutated locus (Cole-Strauss *et al.*, 1996; Yoon *et al.*, 1996). The second method, single-stranded short-fragment homologous replacement (ssSPHR), involves the introduction of short amplicons of nonmutant DNA to the nucleus of mutant cells, and consequent homologous replacement of the mutant allele with the nonmutant counterpart (Kunzelmann *et al.*, 1996; Goncz *et al.*, 1998). While these two methods represent significant potential in the rectification of human disorders involving point mutations, they effect their gene conversion by different cellular mechanisms: chimeraplasty utilizes the mismatch repair mechanism (Fang and Modrich, 1993), while SFHR utilizes the homologous recombination pathway (Hunger-Bertling *et al.*, 1990). As such, these methods may have different potential applications to human disease.

The *mdx* mouse is a useful model of Duchenne muscular dystrophy (DMD). One of several variants has a C-to-T nonsense transition in exon 23 of the X chromosome that causes a truncation of the expressed dystrophin protein (Ryder-Cook *et al.*, 1988; Sicinski *et al.*, 1989; Im *et al.*, 1996). Human DMD is an X-linked recessive disorder affecting 1 in 3000 live-born males. In 65% of boys with DMD, the gene contains gross rearrangements (predominantly frameshift deletions or duplications), while the remaining 35% have a *dys* gene with either nonsense mutations or mutations that affect transcriptional splicing sites (Koenig *et al.*, 1987; Den Dunnen *et al.*, 1989; Liechti-Gallati *et al.*, 1989; Clemens *et al.*, 1992; Kilimann *et al.*, 1992). Gene repair strategies at the *dys* locus therefore need to be able to work in such a manner that enables rectification of frameshifts as well as discrete locus mutations. This will ultimately determine the specific applicability of different gene repair techniques to human DMD.

SFHR has been used to repair a 3-bp microdeletion at the cystic fibrosis transmembrane conductance regulator (CFTR) locus in airway epithelial cells, with successful recombination at 1% of cultured loci (Goncz *et al.*, 1998). Applied to cells containing point mutations in genetic conditions such as DMD, SFHR can fully repair the *dys* gene. Applied to cells containing frameshift mutations, SFHR can be used to insert or delete a single nucleotide to restore the reading frame downstream of the mutation point (Zimmer and Gruss, 1989; Goncz *et al.*, 1998). The potential improvement of correction efficiency was not addressed in previous works beyond the application of bacterial recombination protein RecA, nor was there any attempted *in vivo* application of SFHR reported.

The present study investigates the potential of simple modifications, particularly lipofection reagents, suspension transfection, and multiple applications of SFHR, to improve the efficiency with which SFHR-mediated gene repair occurs in *mdx* myoblasts. Further, this study addresses the potential application of SFHR to promote targeted gene repair by direct injection into *mdx* mouse muscle.

MATERIALS AND METHODS

Mice for SFHR in cell culture

Two male C57BL/10J *mdx* mice, one 12 weeks of age, and the other 9 days old, were obtained (Monash University Animal Services, Melbourne, Australia), exposed to a 12-hr day–night cycle, and fed *ad libitum* until they were required for muscle culture. Another mouse (female C57BL/10 *ScSn/ScSnY* [wild type], 4 months of age) was obtained for cell culture and generation of wild-type DNA for gene repair. All animal handling was performed according to the St. Vincent's Hospital (Fitzroy, Victoria, Australia) Animal Ethics Committee, protocol 26/99, in accordance with the Australian Code of Practice for the Care of Animals for Scientific Purposes National Health and Medical Research Council of Australia, (NHMRC).

Mice for SFHR in vivo

All procedures were approved by the Animal Experimentation Ethics Committee of the University of Melbourne (Melbourne, Victoria, Australia) and conformed to the guidelines for the care and use of experimental animals as described by the NHMRC. Throughout the experimental stages, animals were closely monitored to ensure there was no adverse reaction to the interventions. Four 4-month-old male *mdx* mice (30–40 g) were anesthetized with methohexitone sodium (Brietal [Eli Lilly, Indianapolis, IN], 40–60 mg/kg body weight, intraperitoneal) such that they were unresponsive to tactile stimuli. The tibialis anterior (TA) muscles of the left and right hindlimbs were surgically exposed and maximally injected with a total volume of ~250 μl of 0.5% bupivacaine hydrochloride (Marcain, Astra, North Ryde, NSW, Australia) by one injection each in the proximal, midbelly, and distal regions of the muscle. This was equivalent to, or exceeded, the maximum volume of bupivacaine that each TA muscle could hold, and caused degeneration of the entire muscle mass (our unpublished observations). Intramuscular injection of bupivacaine to a muscle's holding capacity causes complete degeneration of muscle fibers within the first 2 days, followed by complete regeneration (Rosenblatt, 1992). After injection, the small skin incision was closed with Michel clips (Aesculap, Tuttlingen, Germany) and swabbed

with povidone-iodine solution. At the completion of surgery, mice were returned to their cages, where they were fed *ad libitum* and routinely monitored for condition.

Myoblast cell cultures

After asphyxiation with CO_2 and cervical dislocation, skeletal muscle was removed from the *mdx* mice and primary myoblast cultures were prepared essentially as described elsewhere (Austin *et al.*, 1992). The muscle tissue was finely minced with scissors in a small glass petri dish. The myoblast cultures were preplated twice for 1 hr in culture medium containing Ham's F10 (ICN Biomedicals, Costa Mesa, CA) supplemented with 20% fetal calf serum (FCS), basic fibroblast growth factor (bFGF, 2.5 ng/ml), 2 m*M* glutamine (Gln), penicillin (100 units/ml), and streptomycin (100 μg/ml). All cultures were then grown in full growth medium (25-cm^2 flasks) at 37°C and 5% CO_2 with daily half-medium changes until passage at >80% confluence (10^6 cells).

Once cultures reached 80% confluence the cells were detached from the flask, using 0.05% (w/v) trypsin in Hanks' balanced salt solution (HBSS, neutralized by reintroduction to FCS immediately after cell detachment), and counted with a hemocytometer. The cells were divided into two aliquots each containing 5×10^5 cells and pelleted at $400 \times g$ for 10 min, and the medium was aspirated and discarded. One of these pellets was resuspended in full growth medium and grown for subsequent transfection and myotube differentiation; the other was frozen for DNA extraction. The cultures thus intended for transfection were grown to >80% confluence and split for transfection and differentiation.

Preparation of DNA for SFHR, mdx control, and assay

SFHR DNA. DNA was extracted and column purified from the liver of the female wild-type mouse, using the QIAamp tissue DNA extraction protocol according to specification (Qiagen, Valencia, CA), suspended in 15 m*M* Tris-HCl, pH 9.0, and stored at −20°C for DNA analysis. A 603-bp amplicon (amplicon C) was generated by polymerase chain reaction (PCR) with oligonucleotide primers C and D (Fig. 1 and Table 1). The PCRs (100 μl) consisted of 50 ng of total DNA, a 0.4 μ*M* concentration of each primer, 200 μ*M* dNTPs, 1.5 m*M* $MgCl_2$, and 0.5 unit of Bio-X-Act 3'→5' proofreading DNA polymerase system (Bioline/Fisher-Biotech, Perth, Australia) in buffer supplied by the manufacturer. The reactions were subjected to 29 cycles of 92°C for 45 sec (denaturation), 62°C for 2.5 min (annealing/extension) with an initial cycle of 92°C for 2 min and 65°C for 2.5 min (total 30 cycles), using a QuarterBath thermal cycler (Bartelt Instruments, Melbourne, Australia). Amplicon C PCR product was resolved from reagent components by electrophoresis (1% agarose, TAE), followed by ion-exchange chromatography according to manufacturer specifications (QIAEX II system; Qiagen), and suspended in 15 m*M* Tris-HCl, pH 8.8, at a final concentration of 0.1 μg/ul (0.27 pmol/μl).

DNA for mdx *control and assay of* dys *locus repair.* DNA for establishment of control *mdx* template was extracted from the livers of the two male *mdx* mice used for establishing *mdx* cell cultures as described above for wild-type DNA. After gene repair by SFHR, cultures (2×10^5 cells) were harvested and half-passages were used for extraction of DNA (QIAamp), which was then assessed for the presence of wild-type *dys* loci.

Detection of wild-type (repaired) dys *loci by allele-specific PCR*

Allele-specific PCR (as-PCR) was performed with antisense oligonucleotide primer Dys-*wt* AS-01 (3' mismatch for the *mdx* nucleotide) and sense primer B (Table 1 and Fig. 2A). Using this protocol, a 415-bp analytical PCR product was amplified only from wild-type DNA (Fig. 2A). Location of primer B in an intronic region upstream of exon 23 and the *mdx* mutation locus, and in a gene region not present on amplicon C, thus confers chromosomal locus specificity to the as-PCR assay used here. PCR was performed in 25-μl reactions as described above for wild-type DNA, with the exception of a sequential increase in annealing temperature such that *mdx* DNA was not amplified (Fig. 2). Resolution of wild-type and *mdx* templates occurred by application of a 62.5°C annealing temperature.

The tibialis anterior (TA) and vastus lateralis (VL) muscles of the 9-day-old *mdx* mouse were removed and frozen on melting N_2(l)-cooled isopentane for dystrophin immunohistochemistry, skeletal muscle DNA extraction, and RNA extraction. DNA was extracted by the QIAamp Tissue DNA extraction protocol according to the manufacturer instructions (Qiagen), suspended in 15 m*M* Tris-HCl, pH 9.0, and stored at −20°C for DNA analysis by allele-specific PCR and PCR-restriction fragment length polymorphism (RFLP). This DNA (50 ng) and that extracted from transfected and untransfected cultures were subjected to as-PCR as shown in Fig. 2.

Detection of repaired dys *loci by quantitative PCR-RFLP*

PCR-RFLP based on an earlier assay (Shrager *et al.*, 1992; Asselin *et al.*, 1994), was performed on repaired cultures, using a 3'-modified oligonucleotide primer with allele-specific *Mae*III restriction enzyme site acquisition followed by PCR product restriction digestion (Fig. 2B). DNA was extracted from repaired *mdx*, native (unrepaired) *mdx*, and wild-type myoblast cultures, and PCR was performed as described above (for amplicon C) with primers A and D (Fig. 1 and Table 1). Reactions consisted of 50 ng of total DNA, a 0.4 μ*M* concentration of each primer, 200 μ*M* dNTPs, 1.5 m*M* $MgCl_2$, and 2.5 units of Bio-X-Act 3'-5' proofreading DNA polymerase system (Bioline/Fisher-Biotech) in buffer supplied by the manufacturer. The reactions were performed as described for amplicon C to generate a PCR product of 810 bp (amplicon A) exclusive of the SFHR-introduced amplicon C. The PCR product was resolved from reaction components by 1% agarose gel electrophoresis and ion-exchange column chromatography according to the manufacturer specifications (Qiagen) and suspended in 15 m*M* Tris-HCl, pH 8.8, at a concentration of 0.1 μg/μl. A secondary PCR was performed with 100 ng of amplicon A, and a 0.4 μ*M* concentration of each primer (Dys-*Mae* S-01 and Dys-Ex23 AS-01; Table 1 and Fig. 2B) in the presence of [α-^{32}P]dCTP, otherwise as described above for amplicon A. Digestion of this 104-bp amplicon (Dys-*Mae*-104) with *Mae*III with the *mdx* nucleotide at the *dys* locus, produces 60-bp (band S; Fig. 2B) and 44-bp (band B; Fig. 2B) bands. The 3' modification on the Dys-

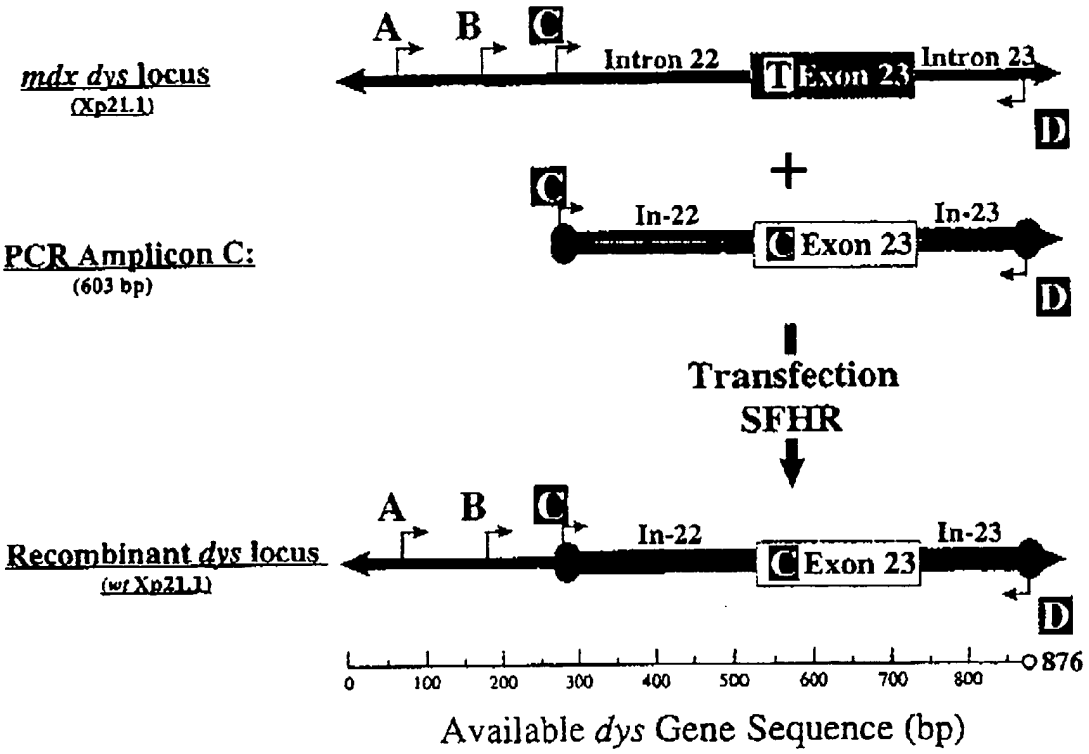


FIG. 1. Strategy for *dys* gene repair by SFHR. Sequence data were obtained for 876 bp of the murine *dys* gene region, including partial sequences of introns 22 and 23 and the entire exon 23 sequence. Oligonucleotide primers A, B, C (sense), and D (antisense) were designed to optimize access to the known sequence at this locus. The oligonucleotide primers were mapped according to this known sequence as shown in Table 1. A 603-bp section of this region was amplified from wild-type C57BL/10 mouse DNA by PCR with primers C and D to generate amplicon C, which contained the wild-type nucleotide (C) at the *mdx* locus. Amplicon C was then used to transfect *mdx* myoblasts and induce homologous replacement of the mutant nucleotide (T) with the wild-type C. Any subsequent PCR amplification with primers A and B of DNA extracted from the transfected *mdx* cultures generated an amplicon that could originate only from the chromosomal locus.

Mae S-01 primer results in site acquisition of a further *Mae*III site only in the presence of the wild-type nucleotide at the *dys* locus. *Mae*III digests the 44-bp *Mae*III digestion product of wild-type Dys-*Mae*-104 PCR product (band B) into two fragments of 20 and 24 bp, respectively (band C; Fig. 2B). Digests were performed at 55°C for 12 hr, using 1 to 2 μg of Dys-*Mae*-104 product generated from repaired mdx, native *mdx*, and wild-type myoblasts, and 10 units of *Mae*III under buffered conditions as specified by the manufacturer (Promega, Madison, WI). The digestion products were resolved on 16% nondenaturing acrylamide gels and visualized by PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Quantification of wild-type loci was performed with ImageQuant software (Molecular Dynamics) and the relative volume densities of bands B (basis, 44 bp) and C (cut, 24/20 bp) as shown in Fig. 2B. Incomplete digestion with the *Mae*III enzyme was quantified by comparison of volume densities of bands S (internal digestion standard, 60 bp) and B in 100% wild-type template controls (Fig. 2B). The possibility that the PCR-RFLP method could generate artifactual positive results owing to carryover of amplicon C in the DNA extracted from the repaired cells was also investigated (Lane *mdx*/C; Fig. 2B). This control was achieved by addition of 10 ng of amplicon C per 1 μl of *mdx* DNA template extracted from the control *mdx* cultures prior to amplicon A amplification by PCR. This ratio of amplicon C and *mdx* DNA represents the ratio expected if the entire 1 μg of amplicon C that was used to transfect the *mdx* cultures was coamplified with the genomic DNA of the repaired cells (100 μl, total volume).

Gene repair at the dys *locus in* mdx *cultures by SFHR*

An amplicon C/LipofectAMINE/Plus reagent complex was formed as follows: heat-denatured (70°C for 3 min) amplicon C (1 μg, 2.7 pmol, 1.6×10^{-3} mol) was added to 100 μl of serum-free Opti-MEM (GIBCO-BRL, Gaithersburg, MD) and allowed to equilibrate to room temperature for 10 min. To this was added 9 μl of Plus reagent (Life Technologies, Rockville, MD), and after thorough mixing the complex was allowed to equilibrate at room temperature for 30 min. To a further 100 μl of Opti-MEM was added 6 μl of LipofectAMINE reagent (Life Technologies), and the mixture was equilibrated at room temperature for 30 min. The two mixtures were then

TABLE 1. OLIGONUCLEOTIDES FOR SHORT-FRAGMENT HOMOLOGOUS REPLACEMENT OF THE *dys* GENE IN *mdx* MOUSE MYOBLASTS[a]

Primer name	Sequence no. (5′)	Primer sequence										Sequence no. (3′)	Direction	Purpose
		3	6	9	12	15	18	21	24	27	30			
A (Dys In22 S-01)	66	CAC	TAT	GAT	TAA	ATG	CTT	GAT	ATT	GAG		92	Sense	Fragment A asPCR
B (Dys In22 S-02)	177	GTT	GAT	TCT	AAA	AAT	CCC	ATG	TTG			200	Sense	Fragment C
C (Dys In22 S-03)	275	GTT	TCA	CTG	TAG	GTA	AGT	AAA	TGT	ATC	AC	303	Sense	Fragments A and C asPCR
D (Dys In23 AS-01)	875	GGC	TTT	TGA	TAT	CAT	CAA	TAT	CTT	TGA	AGG	846	Antisense	
Dys-*wt* AS-01	598	GTC	ACT	CAG	ATA	GTT	GAA	GCC	ATT	TTG		572	Antisense	
Dys-*Mae* S-01[b]	546	CTC	TGC	AAA	GTT	CTT	TGA	AAG	AGT	AA		571	Sense	PCR-RFLP
Dys-Ex23-AS-01	656	CTG	ACA	GAT	ATT	TCT	GGC	ATA	TTT	C		631	Antisense	PCR-RFLP
c3603-AS[c]	3581	CTG	GAT	GCA	AAC	TCA	AGT	TCA	GC			3603	Antisense	RT and RT-PCR
c2801-S[c]	2800	CAC	CCT	ATC	AGA	GCC	AAC	AGC				2821	Antisense	RT-PCR

[a]The sequences of all oligonucleotides, their location with respect to the sequence mapped in Fig. 1, and their purpose in this study are shown.
[b]Sequence modifications from native wild-type sequence are indicated in boldface.
[c]Indicates numbering according to dystrophin cDNA sequence.

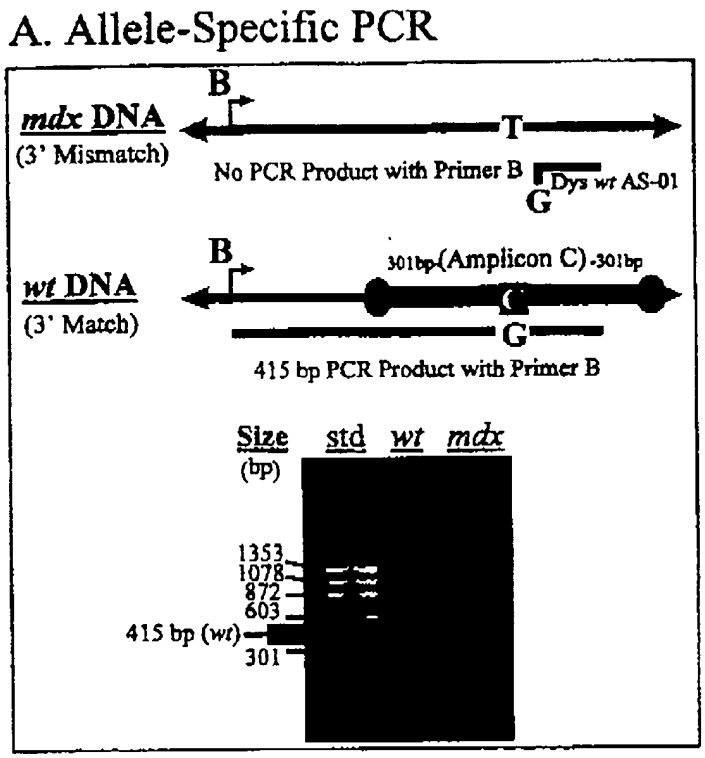


FIG. 2. Detection and quantification of wild-type nucleotide at the *dys* locus. (A) PCR primer Dys-*wt* AS-01 (Table 1) has a 3' mismatch with the mutant T nucleotide at the *mdx* locus. PCR amplification using primers B and Dys-*wt* AS-01 was performed on DNA extracted from skeletal muscle of a 9-day-old *mdx* mouse (from which myoblast cultures were also made; see Fig. 3) and from the wild-type female mouse from which the wild-type amplicon C and wild-type control cultures (Fig. 2A) used in this study were generated. By variation of the annealing temperature during PCR, a 415-bp product can be amplified only from wild-type DNA. The use of primer B in this assay ensures that only chromosomal locus is amplified.

combined and allowed to complex for a further 90 min prior to transfection.

The LipofectAMINE/Plus/amplicon C (6 μl/9 μl/1 μg) complex was introduced to 5×10^5 *mdx* myoblasts (1 cell to 3×10^6 molecules of amplicon C) suspended in 1 ml of Opti-MEM (GIBCO-BRL). The total volume was made up to 3.0 ml with Opti-MEM, and cells were transferred to and allowed to settle in a 25-cm^2 flask (Nunc, Roskilde, Denmark). The transfected myoblasts were propagated at 37°C in 5% CO_2/air for a further 8 hr, after which the medium was supplemented with 2.5 ml of fully supplemented Ham's F10 growth medium without antibiotic or fungicide, containing 20% FCS, bFGF (2.5 ng/ml), and 2 m*M* Gln. These transfected myoblast cultures were grown to >80% confluence in full medium, and split for DNA analysis (culture 1a; Fig. 3) and further transfection/differentiation. This procedure was serially repeated until *mdx* myoblast cultures that had been transfected 0, 1, 2, and 3 times had been generated, sampled for DNA (cultures A, 1a, 1b, and 1c, respectively; Fig. 3), and allowed to differentiate to myotubes for assessment of dystrophin expression. The possibility of artifactual positive results owing to carryover of amplicon C in the DNA extracted from the repaired cells was also investigated (lane A1; Fig. 3). This control was achieved by addition of 10 ng of amplicon C per 1 μl of the DNA (100 μl) template extracted from repaired culture A (*mdx*) prior to as-PCR analysis. This is the maximum ratio of amplicon C and *mdx* DNA possible in the repaired cultures (1a, 1b, and 1c). This would arise only if the entire 1 μg of amplicon C used to transfect culture A was coamplified with the repaired culture (1a, 1b, and 1c) genomic DNA (100 μl, total volume).

Separate experiments were performed with Lipofectin and LipofectAMINE (no Plus reagent; Fig. 4) to assess the relative efficiency of SFHR with different lipofection reagents.

Myotube differentiation was induced by growth of myoblasts in Ham's F10 medium supplemented with 2 m*M* Gln, penicillin (100 units/ml), streptomycin (100 μg/ml), and 7% horse serum (HS) for 2 days, followed by supplemented Ham's F10 medium with 2% HS for a further 7 days. By the end of this incubation, more than 50% of the myoblasts appeared to have differentiated. Growth in 2% HS beyond this point resulted in significant morbidity of cells regardless of whether they had been transfected or not (data not shown).

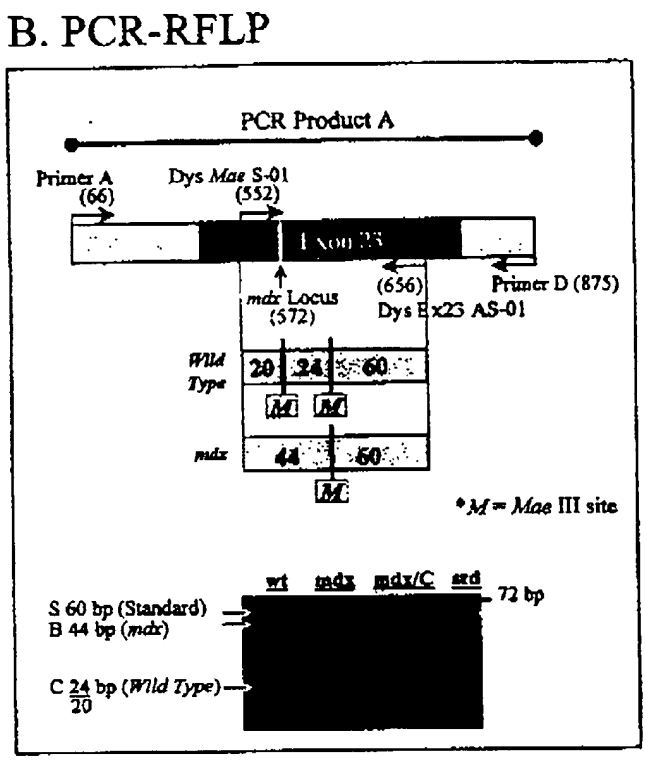


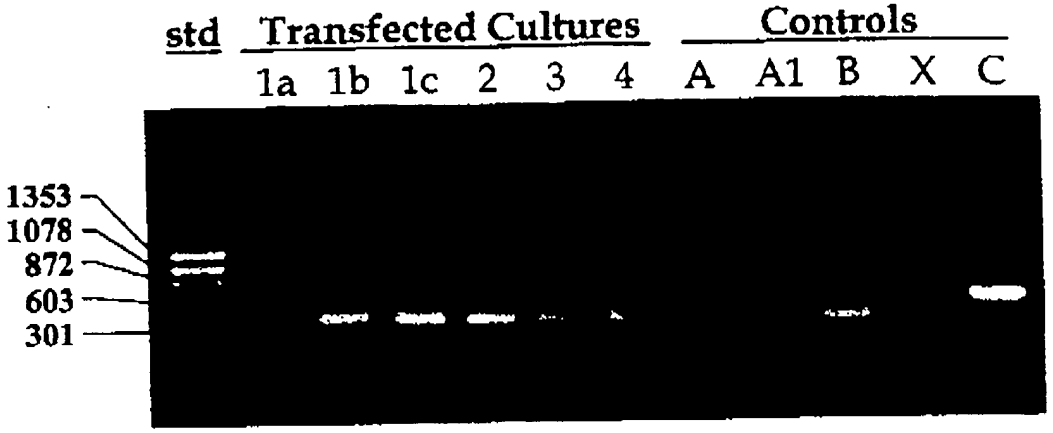
FIG. 2. Detection and quantification of wild-type nucleotide at the *dys* locus. (Continued.) (B) The strategy for PCR-RFLP quantification of repaired cells is shown. PCR was performed with primers A and D to amplify 810 bp (amplicon A) across the repaired region of the *dys* locus in DNA extracted from myoblasts subjected to SFHR. After purification, amplicon A was used as template for a second PCR with primers Dys-*Mae* S-01 and Dys-Ex23 AS-01, the 104-bp product of which was digested with *Mae*III. Primer Dys-*Mae* S-01 is modified near the 3' end such that a *Mae*III site is introduced in the presence of wild-type DNA immediately downstream of the primer-annealing site (Table 1). This results in *Mae*III site acquisition in the 44-bp fragment (B, basis) to generate a doublet band (C, cut) consisting of bands 24 and 20 nucleotides in length. The digestion products are resolved on a 16% nondenaturing acrylamide gel and visualized by PhosphorImager. Incomplete digestion of wild-type control DNA with *Mae*III is shown. The extent of digestion can be estimated by comparison of relative intensities of bands S (60 bp; standard) and B (basis) in 100% wild-type control DNA. The amount of wild-type nucleotide is estimated by comparison of relative intensities of band B (44 bp) and the C (cut, 24/20 bp) doublet. The *mdx*/C control shows that the maximum amount of amplicon C (10 ng) expected to be copurified with DNA from SFHR-repaired cultures will not generate an artifactual result during PCR-RFLP detection when added to *mdx* template prior to amplification of amplicon A.

Assessment of dys *locus expression by RT-PCR*

After differentiation of *mdx* myoblast cultures, mRNA was extracted from 10^5 cells, using the SV RNA extraction protocol (Promega). Total RNA was also extracted from a sample of *mdx* mouse VL, which had been ground on dry ice prior to extraction. First-strand gene-specific reverse transcription was performed on 250 ng of mRNA, using *dys* gene-specific oligonucleotide primer c3603-AS (dystrophin; see Table 1), oligo(dT) (for glyceraldehyde-3-phosphate dehydrogenase [GAPDH]), and Superscript Moloney murine leukemia virus (Mo-MuLV) reverse transcriptase (Life Technologies) under buffered conditions specified by the manufacturer. An 803-bp *dys* transcript product was amplified from this gene-specific cDNA, using c3603-AS and c2801-S primers. Reactions (100 μl) consisting of 0.1 μM concentrations of each primer, a 0.2 m*M* concentration of each dNTP, 2 m*M* $MgCl_2$ and 5 units of *Taq* polymerase (Promega) were subjected to 29 cycles of 93°C for 1 min (denaturation), 57°C for 45 sec (annealing), and 65.5°C for 2 min (extension). A primary 3-min denaturation was performed during the first cycle to ensure optimal first-stage denaturation. The details for GAPDH RT-PCR are described elsewhere (Reardon *et al.*, 2000).

Gene repair at the dys *Locus in* mdx *tibialis anterior by SFHR*

Four days after bupivacaine injection, the mice were anesthetized as described previously. Skin incisions were reopened to facilitate the intramuscular injection of an SFHR cocktail consisting of 25 μg of amplicon C complexed with Lipofectin



Key:

Culture 1a: transfected x1 (12d)
Culture 1b: transfected x2 (12d)
Culture 1c: transfected x3 (12d)
Culture 2: transfected x1 (8d)
Culture 3: transfected x1 (4d)
Culture 4: transfected x1 (*28d)

Control A: Culture 1 (*mdx*)
Control A1: Culture 1/Amplicon C
Control B: Wild Type
Control X: PCR Blank
Control C: Fragment C (No PCR)

FIG. 3. Gene repair at the *mdx* locus by SFHR. Myoblasts were cultured from a 9-day-old *mdx* male (culture A) and a 4-month-old wild-type female (culture B) and DNA was extracted from 10^5 cells. Allele-specific PCR confirmed the genotype of these myoblasts (controls A and B; also in Fig. 2). Controls A1, X, and C were as follows: culture A DNA mixed with 10 ng of amplicon C (prior to PCR), reagent blank (no template DNA), and amplicon C alone, respectively. The 10 ng of amplicon C in the A1 control is the maximum ratio of amplicon C and *mdx* culture A DNA that would be present if all of the amplicon C added during SFHR of culture A were to copurify with the repaired culture (1a, 1b, and 1c) DNA. DNA extracted from culture A after one (culture 1a), two (culture 1b), and three (culture 1c) cycles of SFHR (using LipofectAMINE/Plus) showed the presence of wild-type C at the *mdx* locus of the *dys* gene. SFHR efficiency is visibly improved by SFHR applications subsequent to the first. Cultures 2 and 3 were independent myoblast cultures established from the 9-day-old *mdx* male mouse and repaired once by SFHR. Culture 4 is a totally independent culture (different mouse) that had been subjected to SFHR once 28 days prior to DNA extraction. Numbers in parentheses reflect days after SFHR prior to DNA extraction.

at a ratio of 1:1 (m/v) in 0.9% NaCl (final volume, of 200 μl) into the right TA. An equivalent injection with DNA substituted with saline/Lipofectin vehicle was administered to the left TA muscle to provide a contralateral, no-DNA control. After injection, the skin incisions were reseuled and reswabbed, and the mice were returned to their cages on gaining full consciousness. The mice were killed at 3 weeks after injection and both TAs were removed and frozen on N_2(l)-cooled melting isopentane for immunohistochemical and gene repair analysis.

Immunohistochemical detection of dystrophin expression in myoblasts and muscle

Dystrophin expression in muscle and cultured myotubes was assessed with a polyclonal antibody raised in sheep against a 60-kDa dystrophin fusion protein (kind gift from L. Kunkel, Childrens Hospital and Harvard Medical School, Boston, MA) as described elsewhere (Bower *et al.*, 1997). Dystrophin on the slides was visualized by secondary horseradish peroxidase (HRP)-conjugated rabbit anti-sheep antibody (Dako, Carpinteria, CA), and color was developed with 3,3-diaminobenzidine (DAB), using Sigma Fast DAB tablets according to the manufacturer protocol. Endogenous peroxidase activity was blocked with 0.6% H_2O_2 in phosphate-buffered saline (PBS) prior to incubation with the primary antibody. Myotubes were grown in Labtek II 4-Chamber slides (Nunc) as described above and fixed for 15 min in 0.05% glutaraldehyde–PBS prior to immunohistochemical staining for dystrophin expression.

RESULTS

SFHR gene repair in mdx *mouse myoblasts*

The SFHR strategy used here for repair of the exon 23 C-to-T nonsense transition in *mdx* mouse myoblasts focused on an 876-bp region of the dystrophin locus (*dys*). Representing the entire available sequence data for this region of the *dys* gene, the sequence data encompassed partial nucleotide sequence from introns 22 and 23, and the entire exon 23 sequence (Fig. 1).

The location of primers A and B outside of amplicon C (generated by primers C and D) provided access to the chromosomal locus in repaired cells exclusive of the introduced amplicon C (Figs. 1 and 2A). This forms the basis for a novel

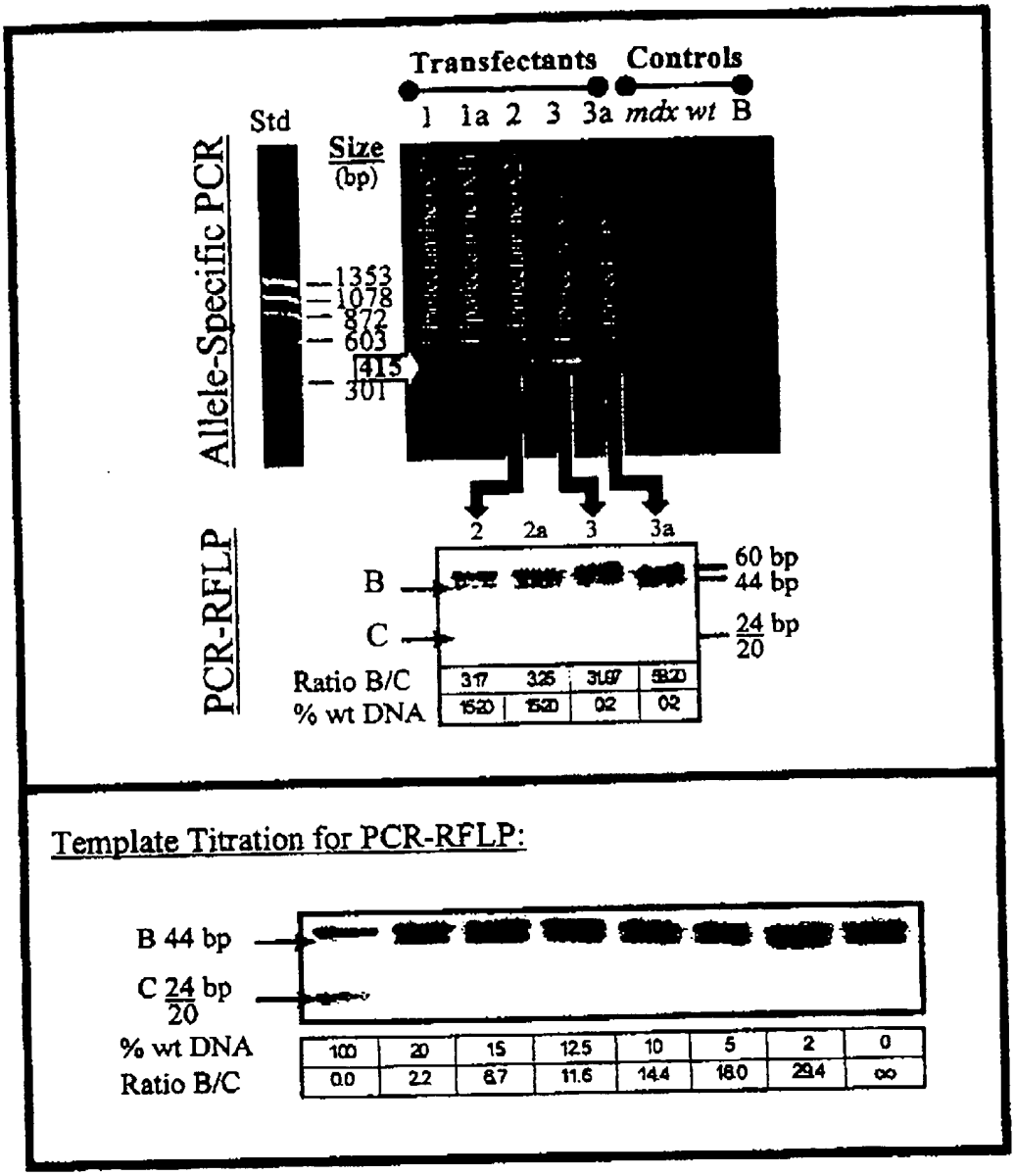


FIG. 4. SFHR II: Double application and lipofection variation. Myoblast cultures were generated from a 4-month-old *mdx* mouse, and then divided into five tandem cultures (*mdx*, 1, 1a, 3, and 3a). SFHR was performed on cultures 1 and 1a once, using LipofectAMINE (no Plus reagent), and on cultures 3 and 3a once, using Lipofectin. Culture *mdx* was grown without SFHR. Cultures 1 and 1a were grown to passage, with one passage from each being grown further and the other being subjected to SFHR one further time to generate cultures 2 and 2a (SFHR = twice). Cultures 3 and 3a were passaged, with one passage harvested for DNA analysis and the other for myotube differentiation. Cultures 1, 1a, 2, and 2a were likewise passaged to generate a DNA fraction and a differentiated fraction for *dys* gene expression analysis. Allele-specific PCR shows that cultures 1 and 1a had minimal levels of repair after a single cycle of SFHR (LipofectAMINE), which was visibly improved by a further cycle (cultures 2 and 2a; 2a as-PCR not shown). Alternatively, as-PCR revealed much more significant levels of gene repair after a single SFHR cycle using Lipofectin (cultures 3 and 3a). By titration of wild-type and *mdx* DNA template and application of PCR-RFLP (*bottom*), phosphorimager data verified the semiquantitative as-PCR result, and showed that 15 to 20% of *dys* loci in cultures 2 and 2a and up to 2% of loci in cultures 3 and 3a had been converted to wild-type at the *mdx* position. Cultures 1 and 1a could not be quantified by PCR-RFLP, which indicates that repair levels may have been as low as 5×10^{-4}% of loci in the initial SFHR cycle (data not shown).

allele-specific PCR (as-PCR) strategy that is able to resolve one wild-type *dys* locus from 10^5 *mdx* loci (data not shown). The as-PCR strategy utilizes primer B with primer Dys-*wt* AS-01, which has a 3′ mismatch with the mutant T nucleotide at the *mdx* locus and results in differential amplification of wild-type template in a mixture of *mdx* and wild-type DNA (Fig. 2A). This high-resolution technique was designed specifically for detection of the low-level (~1%) gene repair expected from SFHR (Goncz *et al.*, 1998) in the 10^5 cells grown. Because of the exponential amplification of template, using this method, the as-PCR strategy could be considered only semiquantitative. PCR-RFLP using allele-specific *Mse*III digestion was able to provide quantitative analysis of *dys* loci in repaired cell cultures (Fig. 2B). In contrast to the exponential nature of allele-specific PCR, PCR-RFLP has a linear range of wild-type locus detection in repaired (heterogeneous) cultures and can therefore be used as a quantitative measure.

Prior to myoblast culture, as-PCR analysis of DNA extracted from the tibialis anterior (TA) of a 9-day-old male *mdx* mouse showed a total absence of wild-type C at the *mdx* locus (Fig. 2A, *mdx* lane). Likewise, cells cultured from the remaining muscle of the same *mdx* mouse were exclusively of the *mdx* genotype (Fig. 3, lanes A and A1). After a single transfection, using LipofectAMINE/Plus reagent, of three such cultures established from the *mdx* mouse, as-PCR was able to detect successful replacement of mutant T nucleotide with the wild-type C at the *mdx* locus in 0.1 to 5×10^{-4}% of cells (Fig. 3, cultures 1a, 2, and 3), which persisted for at least 28 days postrepair (Fig. 3, culture 4). After two further serial applications of SFHR (cultures 1b and 1c; Fig. 3), improved repair efficiency was evident on agarose gels compared with just a single application of SFHR. This result was confirmed in further independent experiments involving myoblasts cultured from a single 4-month-old male *mdx* mouse (Fig. 4). In the latter experiment, SFHR efficiency in cultures transfected once with LipofectAMINE (1 and 1a) was improved by a subsequent transfection (cultures 2 and 2a; Fig. 4). Furthermore, SFHR efficiency with Lipofectin (Fig. 4, cultures 3 and 3a) and LipofectAMINE/Plus was better after single applications than with Lipofectamine alone. This result indicates that SFHR efficiency may be improved by variation of the transfection method and by multiple applications. In the second experiment (Fig. 4) the multiply transfected cultures (2 and 2a) were assayed for wild-type loci by PCR-RFLP, and showed a repair frequency of 15 to 20%, using ImageQuant software (Molecular Dynamics). Of the remaining cultures transfected in the second experiment (Fig. 4), cultures 3 and 3a displayed the presence of wild-type loci in 1 to 2% of loci, using the PCR-RFLP quantitative technique. Cultures 1 and 1a contained wild-type loci beyond the resolution limit of the PCR-RFLP strategy, but just detectable with the as-PCR technique and therefore in between 5×10^{-4} and 0.1% of cells. The results are in good agreement with SFHR gene repair frequencies achieved in pivotal SFHR experiments by others (Kunzelmann *et al.*, 1996; Goncz *et al.*, 1998).

Expression of repaired dys *Loci*

RT-PCR. After passage harvesting for DNA extraction, the remainders of cultures 1 through to 3a were allowed to undergo myotube formation. After 7 days of differentiation, Cultures 1, 1a, 3, and 3a and cultures 2 and 2a, respectively, were pooled to establish two composite repaired myotube sources and centrifuged, and pellets were frozen for RNA analysis. RNA extracted from these composite myotubes represented single and double SFHR applications, respectively. The RNA obtained from these cultures was analyzed by RT-PCR for dystrophin transcript as described earlier and shown in Fig. 5. No dystrophin transcript was detected in these myotube cultures (Fig. 5A, lane 2 & 2a). Neither was there any dystrophin transcript detected in cultures subjected to sham lipofection (Fig. 5A, lane *mdx*-C), where no SFHR was performed (no amplicon C). In RT-PCR performed on RNA extracted from *mdx* and wild-type muscle, dystrophin transcript was detected in amounts of RNA corresponding to that used in the myotube culture RNA (Fig. 5A, lanes *wt-m* and *mdx-m*). RNA integrity in the muscle and myotube extractions was assessed by RT-PCR amplification of GAPDH transcript (equal quantities) from the *wt-m* and 2/2a myotube extracts (Fig. 5B). Both the muscle and myotube RNA could equally be used for RT-PCR. The cultures containing the repaired genes were thus shown not to be expressing detectable levels of dystrophin transcript.

Immunohistochemistry. Aliquots of the passaged cells were grown and allowed to undergo differentiation on chamber slides. Cell numbers diminished markedly during the differentiation process and differentiation beyond 7 days left few cells/tubes on the slide. This effect was considerably more marked in multiple SFHR application cultures, where again cultures were significantly depleted by 7 days of differentiation. Immunohistochemistry of the myotubes on these slides revealed no dystrophin expression that differed from untreated *mdx* myotubes.

SFHR gene repair in mdx *mouse tibialis anterior*

Three weeks after injection of the SFHR-wild-type and SFHR-vehicle cocktails into the right and left TAs of the four male *mdx* mice, the mice were killed by cervical dislocation and TAs removed for immunohistochemistry and DNA screening. The frozen TAs were sectioned and immunohistochemically stained with anti-dystrophin antibody. As for *in vitro* SFHR, no differences were observed between amplicon- and vehicle-injected TAs in the numbers or extent of fibers staining positive for dystrophin.

After immunohistochemical examination, DNA was extracted from these tissues and showed that all four TAs injected with the DNA cocktail showed repair at the *dys* locus. In contrast, the DNA from contralateral TAs injected with saline cocktail lacked evidence of repair at the *dys* locus (Fig. 6). Application of the PCR-RFLP technique to these tissues did not reveal sufficient DNA repair to visualize by this method. This result suggests that *in vivo* SFHR repair of the *dys* locus occurred at a frequency somewhere between 5×10^{-4} and 0.1% of the regenerating cells in the injected *mdx* TAs. The relatively low frequency of gene repair achieved *in vivo*, while promising, underlies the lack of detectable dystrophin expression by immunohistochemistry both for *in vivo* applications presented here, as well as in the *in vitro* application of SFHR.

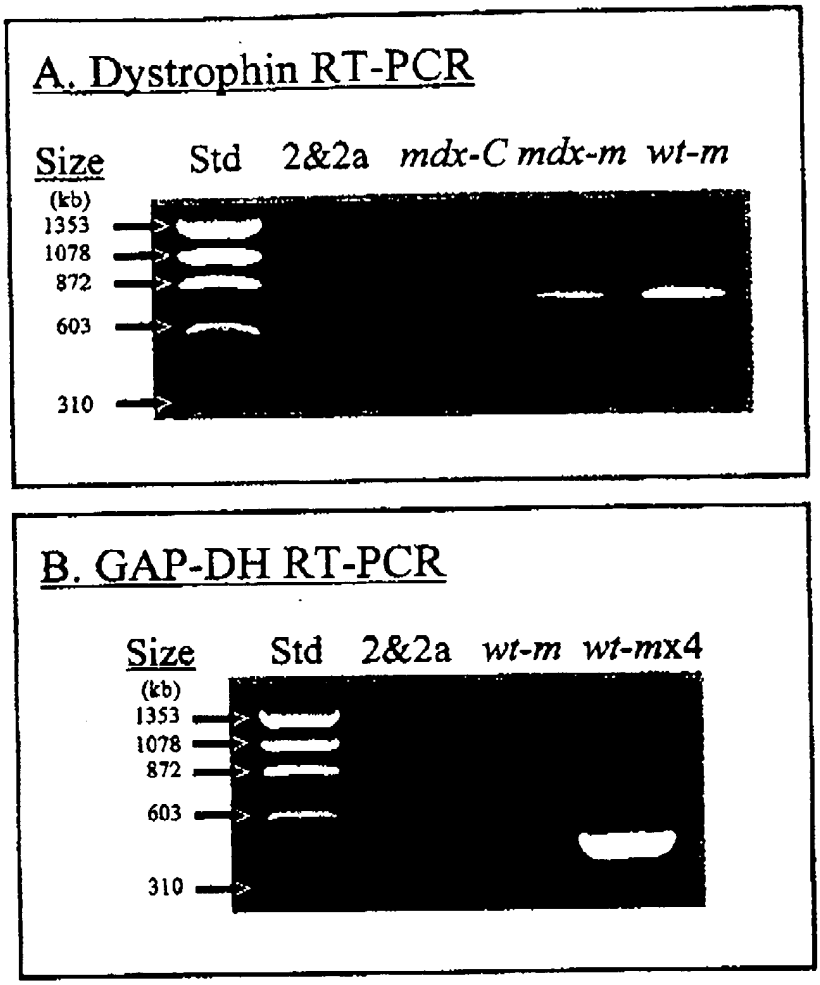


FIG. 5. Dystrophin gene expression in repaired cultures. Myoblasts, cultured from a 9-day-old male *mdx* mouse, were subjected to two cycles of SFHR (cultures 2 and 2a; Fig. 4), and a subpassage was allowed to differentiate into myotubes. RNA was extracted from these cultures after 7 days of differentiation and pooled, and first-strand RT-PCR was performed with *dys* gene-specific (c3603–AS and c2801–S; Table 1) and poly(A)-specific oligonucleotide primers. The cDNA products were used for amplifying sections of *dys* (A) and GAPDH (B) transcripts, respectively, by PCR. Analysis of *dys* transcript was performed on equal amounts of template (see Materials and Methods) generated from the twice-transfected (2&2a) and sham-transfected (transfected without any DNA; *mdx-C*) myotubes, *mdx* muscle (*mdx-m*), and wild-type muscle (*wt-m*). After 7 days, neither the transfected (2&2a) nor sham-transfected (*mdx-C*) myotubes expressed dystrophin transcript. An equal amount of mRNA from *mdx* (*mdx-m*) and wild-type (*wt-m*) muscle, on the other hand, was shown to express detectable dystrophin transcripts by this method. The integrity of mRNA in the transfected cultures (2&2a) was compared with that in the muscle, using GAPDH expression as a standardizing factor (B). These RT-PCR experiments showed that in the presence of equal quantities of starting mRNA, the GAPDH transcript was equally detectable in the cultured cells as in the muscle. The *wt-m* × 4 lane shows the effect of a 4-fold increase in *wt-m* mRNA at the start of the RT.

DISCUSSION

This study investigated possible application of short-fragment homologous replacement (SFHR) to mutations involved in genetic neuromuscular conditions. In addition to primary application of SFHR to the *mdx dys* locus nonsense mutation, this study focused on SFHR gene repair efficiency improvement and *in vivo* application. The animal model used in this work was one from several variants of the *mdx* mouse model of Duchenne muscular dystrophy (DMD). The model expresses a C-to-T nonsense transition in dystrophin gene (*dys*) exon 23 of the X chromosome that causes a C-terminal truncation of the

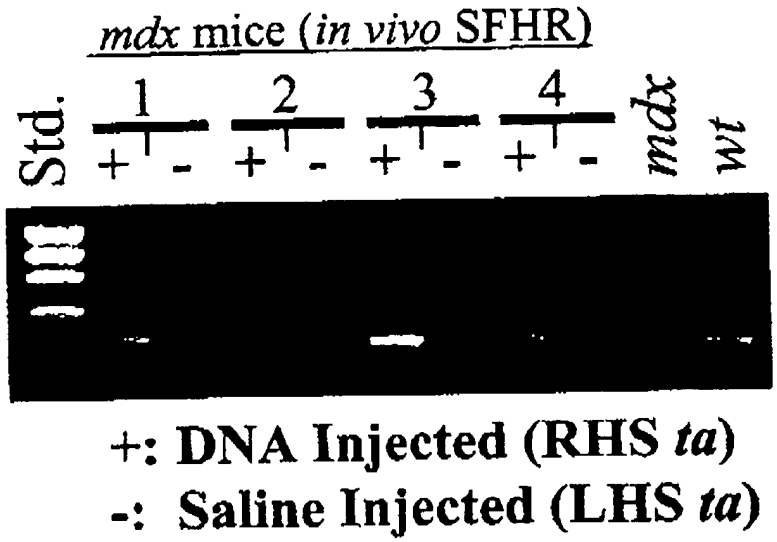


FIG. 6. Dystrophin gene repair by SFHR in *mdx* tibialis anterior. Right tibialis anterior (TA) from four 12-week-old male *mdx* mice was injected with SFHR cocktail after bupivacain treatment, while left TA was injected with saline/vehicle. The mice were then killed and both TAs were dissected out, snap frozen, and analyzed for dystrophin expression by anti-dystrophin immunohistochemistry. After histochemistry, the remaining TA was ground to a powder and the DNA was extracted. Allele-specific PCR was applied to the DNA from both sides and showed evidence of repair in the right TAs compared with the left TAs. Repair was not sufficient to allow evaluation of repaired gene dystrophin expression by RT-PCR-RFLP.

expressed dystrophin protein (Ryder-Cook *et al.*, 1988; Sicinski *et al.*, 1989).

Dystrophin gene repair in this study was achieved in 15 to 20% of cultured *mdx* myoblasts and in somewhere between 5×10^{-4} and 0.1% of muscle cells in tibialis anterior of male *mdx* mice *in vivo*. These *in vitro* repair frequencies are higher than in early pioneering SFHR studies that reported repair of the common ΔF508 CFTR locus microdeletion in 1% of airway epithelial cells (Kunzelmann *et al.*, 1996; Goncz *et al.*, 1998). This finding, however, cannot be considered comparative because of the use of different cell types in the early studies compared with those used here. Repair of the *mdx dys* locus by SFHR in this study now joins an alternative strategy involving chimeraplasty, applied to muscle in the *mdx* mouse (Rando *et al.*, 2000) and in the *GRMD* (golden retriever muscular dystrophy) dog (Bartlett *et al.*, 2000) models of DMD.

Chimeraplasty involves activation of mismatch repair mechanisms to effect gene repair, whereas SFHR utilizes homologous recombination pathways. Of these two methods, SFHR is the only one to date that has been applied to insertion of small nucleotide sequences (3 bp) to repair disease-related mutations (Kunzelmann *et al.*, 1996; Goncz *et al.*, 1998). The *mdx* mouse and *GRMD* dog models show a pathophysiology similar to that of human DMD at the genetic level. The *GRMD* dog has a splice junction mutation at intron 6 that causes a frameshift deletion at the secondary transcript level (Sharp *et al.*, 1992). The *mdx* mouse used in this study expresses a nonsense mutation. Gene repair by insertion of trinucleotide sequence at the CFTR locus by others using SFHR, however, suggests that frameshift mutations could be repaired by the insertion or deletion of a single nucleotide at or downstream of the deletion interface.

In contrast to both reports of *dys* gene repair by chimeraplasty, as well as SFHR gene repair of the ΔF508 CFTR mutation in airway epithelial cells, the expression of repaired *dys* loci in this study was not detectable at the protein or transcript level. In view of the high and low repair efficiencies observed here *in vitro* and *in vivo*, respectively, this important factor bears careful consideration. Both studies of chimeraplastic repair at the *dys* locus were *in vivo* studies, and showed higher *in vivo* repair levels than those demonstrated in this report. In the *mdx* study (Rando *et al.*, 2000), expression of the 1 to 2% repaired *dys* loci was demonstrable only by Western analysis after immunoprecipitation of the expressed dystrophin and by use of revertant fiber-exclusive antibody, while the *GRMD* dog repair was evident in up to 10% of transcripts analyzed. Both these studies report at least two orders of magnitude higher *in vivo* *dys* gene repair than achieved in this study.

Alternatively, the high *in vitro* repair frequencies achieved here in double SFHR applications were easily detectable at the *dys* gene level by the quantitative PCR-RFLP method (Fig. 4). While expression of SFHR-repaired CFTR loci was demonstrable in cultured airway epithelial cells (Kunzelmann *et al.*, 1996; Goncz *et al.*, 1998), demonstration of repaired *dys* locus expression in this study required differentiation of myoblasts under limited serum conditions. This differentiation was observed by phase-contrast microscopy in up to 50% of cells in repaired and control cultures, and yet RNA analysis of the differentiated *mdx* cultures showed that no dystrophin transcript was being expressed by these myotubes (Fig. 5) at harvest. The mechanism underlying the lack of *dys* gene expression in these myotubes is somewhat unclear, but is most likely directly or indirectly related to the transfection/lipofection process, which

was observed to have adverse effects on the viability of cells. While there is a possibility that the SFHR process produces deleterious gene modifications by nonspecific integration in addition to the observed dystrophin gene repair, the experiments performed here are not capable of resolving this issue. This possibility would not, however explain the lack of dystrophin transcript per se in the repaired cultures (Fig. 5). One possible explanation may be that the 7-day differentiation of the cultures was not sufficient to allow adequate expression of dystrophin. This possibility is supported by a lack of *dys* gene transcript in sham-transfected *mdx* cultures (*mdx*-C; Fig. 5) and by observations that *dys* gene transcripts are not detected 7 days after differentiation (Radojevic *et al.*, 2000). A further possibility, however also remains that lipofection-mediumted SFHR of cells may adversely affect their subsequent ability to express dystrophin. Support for this exists in the inability to cultivate the repaired myotubes to a significant extent beyond the 7-day window observed after differentiation. The lack of *dys* gene transcript was further confirmed by immunohistochemistry, where no differences were observed between untreated and treated *mdx* cultures. Muscle immunohistochemistry likewise was unable to resolve dystrophin expression in SFHR-treated muscles compared with untreated muscles. This result reflects the extremely low repair frequency achieved *in vivo*.

In this study, three lipofection reagents were used. SFHR using LipofectAMINE was unable to generate significant levels of gene correction after a single application. Significant levels of gene repair were evident only after two applications of LipofectAMINE-medated SFHR (Fig. 3). Alternatively, Lipofectin was able to generate a higher level of SFHR gene repair efficiency after the first application than LipofectAMINE. This improvement was offset, however, by increased senescence and morbidity associated with myoblasts transfected with Lipofectin. In general, they did not survive multiple applications very well. Use of the LipofectAMINE/Plus reagent system resulted in an intermediate compromise between efficiency and relatively low senescence/morbidity effects compared with the Lipofectin reagent and in much better first-application repair efficiency than LipofectAMINE alone. It is thus possible that combined applications of other lipofectants or transfectants such as polyethyleneimine (PEI) or dendrimers may be of further benefit to SFHR efficiency *in vitro*. Likewise, application of nonchemical techniques such as electroporation or microinjection may further improve SFHR and subsequent cell viability.

The prospect of direct *in vivo* SFHR is favorable for application in genetic muscle disorders such as DMD. Unlike the *in vitro* experiments, *in vivo* SFHR *mdx* locus repair demonstrated here was detectable only by the high-resolution allele-specific PCR (as-PCR) method (Fig. 6). This places the frequency of repaired *mdx* loci at between 5×10^{-4} and 0.1% in the transfected muscles. The demonstration of low-level *mdx dys* locus repair *in vivo* per se gives rise to the possibility that further efficiency improvement may see the application of SFHR gene correction directly in muscle without any associated *in vitro* cellular process. Such improvements may take the form of whole muscle electroporation, which has shown great success in transfecting whole muscle with reporter plasmid (Mir *et al.*, 1999). Under these conditions, application of myotoxic agents as used here, may or may not be required for gene repair.

The work presented in this article reports targeted repair of the C-to-T nonsense transition at exon 23 of the *dys* gene in the *mdx* mouse. Genetic correction of cells by SFHR or otherwise has the potential benefit that repaired cells differ in genetic character by only a single nucleotide locus from the host cells. With further improvements to *in vivo* and *in vitro* gene repair processes, SFHR and other gene repair techniques may find some application in the rectification of genetic defects in neuromuscular diseases.

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The authors dedicate this manuscript to the memory of Dr. Lawrence Austin, who provided much inspiration to all who knew and worked with him. Lawrie passed away at home on Tuesday, November 7, 2000, at 5.30 P.M. His wisdom, strength, and knowledge were unique and will be remembered by all.

REFERENCES

ASSELIN, I., TREMBLAY, M., VILQUIN, J.T., GUERETTE, B., and TREMBLAY, J.P. (1994). Polymerase chain reaction-based assay to assess the success of myoblast transplantation in *mdx* mice. Transplant. Proc. 26, 3389.

AUSTIN, L., BOWER, J., KUREK, J., and YAKAKIS, N. (1992). Effects of leukaemia inhibitory factor and other cytokines on murine and human myoblast proliferation. J. Neurol. Sci. 112, 185–191.

BARTLETT, R.J., STOCKINGER, S., DENIS, M.M., BARTLETT, W.T., INVERARDI, L., LE, T.T., MAN, N., MORRIS, G.E., BOGAN, D.J., METCALF-BOGAN, J., and KORNEGAY, J.N. (2000). *in vivo* targeted repair of a point mutation in the canine dystrophin gene by a chimeric RNA/DNA oligonucleotide. Nat. Biotechnol. 18, 615–622.

BOWER, J., WHITE, J., KUREK, J., MULDOON, C.M., and AUSTIN, L. (1997). The role of growth factors in myoblast transfer therapy. Basic Appl. Myol. 7, 177–186.

CAPECCHI, M.R. (1989a). The new mouse genetics: Altering the genome by gene targeting. Trends Genet. 5, 70–76.

CAPECCHI, M.R. (1989b) Altering the genome by homologous recombination. Science 244, 1288–1292.

CLEMENS, P.R., WARD, P.A., CASKEY, C.T., BULMAN, D.E., and FENWICK, R.G. (1992). Premature chain termination mutation causing Duchenne muscular dystrophy. Neurology 42, 1775–1782.

COLE-STRAUSS, A., YOON, K., XIANG, Y., BYRNE, B.C., RICE, M.C., GRYN, J., HOLLOMAN, W.K., and KMIEC, E.B. (1996). Correction of the mutation responsible for sickle cell anemia by an RNA–DNA oligonucleotide. Science 273, 1386–1389

DEN DUNNEN, J.T., GROOTSCHOLTEN, P.M., BAKKER, E., BLONDEN, L.A., GINJAAR, H.B., WAPENAAR, M.C., VAN PAASSEN, H.M., VAN BROECKHOVEN, C., PEARSON, P.L., and VAN OMMEN, G.J. (1989). Topography of the Duchenne muscular dystrophy (DMD) gene: FIGE and cDNA analysis of 194 cases reveals 115 deletions and 13 duplications. Am. J. Hum. Genet. 45, 835–847.

FANG, W.H., and MODRICH, P. (1993). Human strand-specific mis-

match repair occurs by a bidirectional mechanism similar to that of the bacterial reaction. J. Biol. Chem. 268, 11838–11844.

FOLGER, K.R., WONG, E.A., WAHL, G., and CAPECCHI, M.R. (1982). Patterns of integration of DNA microinjected into cultured mammalian cells: Evidence for homologous recombination between injected plasmid DNA molecules. Mol. Cell. Biol. 2, 1372–1387.

GONCZ, K.K., KUNZELMANN, K., XU, Z., and GRUENERT, D.C. (1998). Targeted replacement of normal and mutant CFTR sequences in human airway epithelial cells using DNA fragments. Hum. Mol. Genet. 7, 1913–1919.

HUNGER-BERTLING, K., HARRER, P., and BERTLING, W. (1990). Short DNA fragments induce site specific recombination in mammalian cells. Mol. Cell. Biochem. 92, 107–116.

IM, W.B., PHELPS, S.F., COPEN, E.H., ADAMS, E.G., SLIGHTOM, J.L., and CHAMBERLAIN, J.S. (1996). Differential expression of dystrophin isoforms in strains of *mdx* mice with different mutations. Hum. Mol. Genet. 5, 1149–1153.

KILIMANN, M.W., PIZZUTI, A., GROMPE, M., and CASKEY, C.T. (1992). Point mutations and polymorphisms in the human dystrophin gene identified in genomic DNA sequences amplified by multiplex PCR. Hum. Genet. 89, 253–258.

KOENIG, M., HOFFMAN, E.P., BERTELSON, C.J., MONACO, A.P., FEENER, C., and KUNKEL, L.M. (1987). Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. Cell 50, 509–517.

KUNZELMANN, K., LEGENDRE, J.Y., KNOELL, D.L., ESCOBAR, L.C., XU, Z., and GRUENERT, D.C. (1996). Gene targeting of CFTR DNA in CF epithelial cells. Gene Ther. 3, 859–867.

LIECHTI-GALLATI, S., KOENIG, M., KUNKEL, L.M., FREY, D., BOLTSHAUSER, E., SCHNEIDER, V., BRAGA, S., and MOSER, H. (1989). Molecular deletion patterns in Duchenne and Becker type muscular dystrophy. Hum. Genet. 81, 343–348.

MANSOUR, S.L., THOMAS, K.R., DENG, C.X., and CAPECCHI, M.R. (1990). Introduction of a *lacZ* reporter gene into the mouse *int-2* locus by homologous recombination. Proc. Natl. Acad. Sci. U.S.A. 87, 7688–7692.

MIR, L.M., BUREAU, M.F., GEHL, J., RANGARA, R., ROUY, D., CAILLAUD, J.M., DELAERE, P., BRANELLEC, D., SCHWARTZ, B., and SCHERMAN, D. (1999). High-efficiency gene transfer into skeletal muscle mediated by electric pulses. Proc. Natl. Acad. Sci. U.S.A. 96, 4262–4267.

RADOJEVIC, V., LIN, S., and BURGUNDER, J.M. (2000). Differential expression of dystrophin, utrophin, and dystrophin-associated proteins in human muscle culture. Cell Tissue Res. 300, 447–457.

RANDO, T.A., DISATNIK, M.H., and ZHOU, L.Z. (2000). Rescue of dystrophin expression in *mdx* mouse muscle by RNA/DNA oligonucleotides. Proc. Natl. Acad. Sci. U.S.A. 97, 5363–5368.

REARDON, K.A., KAPSA, R.M., DAVIS, J., KORNBERG, A.J., AUSTIN, L., CHOONG, P., and BYRNE, E. (2000). Increased levels of leukemia inhibitory factor mRNA in muscular dystrophy and human muscle trauma. Muscle Nerve 23, 962–966.

ROSENBLATT, J.D. (1992). A time course study of the isometric contractile properties of rat extensor digitorum longus muscle injected with bupivacaine. Comp. Biochem. Physiol. Comp. Physiol. 101, 361–367.

RYDER-COOK, A.S., SICINSKI, P., THOMAS, K., DAVIES, K.E., WORTON, R.G., BARNARD, E.A., DARLISON, M.G., and BARNARD, P.J. (1988). Localization of the *mdx* mutation within the mouse dystrophin gene. EMBO J. 7, 3017–3021.

SHARP, N.J., KORNEGAY, J.N., VAN CAMP, S.D., HERBSTREITH, M.H., SECORE, S.L., KETTLE, S., HUNG, W.Y., CONSTANTINOU, C.D., DYKSTRA, M.J., and ROSES, A.D. (1992). An error in dystrophin mRNA processing in golden retriever muscular dystrophy, an animal homologue of Duchenne muscular dystrophy. Genomics 13, 115–121.

SHRAGER, J.B., NAJI, A., KELLY, A.M., and STEDMAN, H.H. (1992). A PCR-based assay for the wild-type dystrophin gene transferred into the *mdx* mouse. Muscle Nerve 15, 1133–1137.

SICINSKI, P., GENG, Y., RYDER-COOK, A.S., BARNARD, E.A., DARLISON, M.G., and BARNARD, P.J. (1989). The molecular basis of muscular dystrophy in the *mdx* mouse: A point mutation. Science 244, 1578–1580.

THOMAS, K.R., and CAPECCHI, M.R. (1987). Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell 51, 503–512.

YOON, K., COLE-STRAUSS, A., and KMIEC, E.B. (1996). Targeted gene correction of episomal DNA in mammalian cells mediated by a chimeric RNA·DNA oligonucleotide. Proc. Natl. Acad. Sci. U.S.A. 93, 2071–2076.

ZIMMER, A., and GRUSS, P. (1989). Production of chimaeric mice containing embryonic stem (ES) cells carrying a homoeobox *Hox 1.1* allele mutated by homologous recombination. Nature 338, 150–153.

Address reprint requests to:
Dr. Robert Kapsa
Melbourne Neuromuscular Research Institute
Department of Clinical Neurosciences
St. Vincent's Hospital
41 Victoria Pde.
Fitzroy, Victoria 3065, Australia

E-mail: rkapsa@ariel.ucs.unimelb.edu.au

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